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Joint effects of three plant protection products to the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*

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ABSTRACT

The effects of simultaneous application of plant protection products are of concern since the uses of different products pose an additional risk to non-target soil organisms. The effects of binary combinations of dimethoate, glyphosate and spirodiclofen, an insecticide an herbicide and an acaricide, on the avoidance behaviour of the terrestrial isopod *Porcellionides pruinosus* and the reproductive effort of *Folsomia candida* were assessed using the two reference models of concentration addition (CA) and independent action (IA). Results of single exposure to the three pesticides indicated a clear dose related avoidance response of the isopods in the highest concentrations tested of the three as well as a strong decrease in collembolan adult survival and concomitant number of juveniles produced. In the combined experiments, antagonism was found in 7 out of the 12 combinations, four combinations followed the reference models, and only in one combination synergism was detected (lower doses of glyphosate and spirodiclofen applied to *P. pruinosus*). In conclusion, it seems that mixing and applying these products, at the recommended field application rate, does not lead to enhanced toxicity, hence limited risk is associated with the joint application of these pesticides.

1. Introduction

Chemical risk evaluation is based on the assessment of the effects of single chemicals to single test-species although in real scenarios organisms are exposed to mixtures of chemicals (Baas et al., 2007). In agricultural fields, for example, several pesticides are applied at the same time or in consecutive days, which could pose an additional risk to non-target organisms (Junghans et al., 2006). Therefore it seems pertinent to evaluate possible detrimental effects that may arose from the combined application of pesticides to non-target edaphic organisms (Larsen et al., 2003).

Two different concepts, imported from pharmacological studies, are used in the ecotoxicological assessment of chemical mixtures: concentration addition (CA), first described by Loewe and Muischnek (1926), and independent action (IA), described by Bliss (1939). The main difference among these two concepts is related with chemicals mode of action, since CA is used for similarly acting substances whereas IA is used for dissimilarly acting substances, although both models assume that no interaction between the two substances in the mixture takes place (Hewlett and Plackett, 1959). Both conceptual models (CA and IA) were fitted to the isopods avoidance behaviour data and collembolan reproductive output data gathered from the three binary mixtures performed. The strategy followed in this study was based on the fact that the specific molecular mode of action of these three pesticides to the isopod *Porcellionides pruinosus* and *Folsomia candida* were unknown, thus in the absence of a clear and precise knowledge of the mechanism of toxicity that one chemical may exert to the target organism, CA and IA can be used as equally valid reference models (Gomez-Eyles et al., 2009). In 2005, Jonker et al. described the MixTox model as a tool that can be used to derive patterns of response of binary mixtures. As a first approach this tool fit ecotoxicity data to the conceptual models (the CA or the IA) and then evaluated if there are any deviations for synergism/antagonism or dose level or ratio dependencies (i.e. depending on low or high doses, or dependent on the ratio of the chemicals in the mixture, respectively).

In soil ecosystems terrestrial isopods can be found in the soil surface layer and are responsible for the decomposition of litter contributing decisively for nutrient recycling in edaphic ecosystems (Zimmer, 2002). Their importance in influencing microbial respiration as well in increasing the availability of macronutrients in the upper soil layer has been well established (Kautz and Topp, 2000). So, the evaluation of possible impairments in the isopods populations due to applications of pesticides is of critical importance, considering the essential role in soil ecosystems maintenance that these organisms have (Loureiro et al., 2005).

The springtail *F. candida* is a useful indicator organism because it has a short life cycle, is present in high densities in terrestrial ecosystems and has a widespread distribution through all Europe (Skovlund et al., 2008) and can give an earlier indication of

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ecosystem disturbance (Jänsch et al., 2005). This species is parthenogenetic, easy to culture and has a relatively short generation time, so it is possible to study different individual and population parameters in one experiment (Greenslade and Vaughan, 2003). It contributes to the decomposition processes in soil by grazing on bacteria and fungi and breaking down organic matter (Petersen, 2002) so it is important to understand if agricultural practices may affect their population levels (Eaton, 2006).

Several tests have been standardized and are available to evaluate chemical exposure as the earthworms and springtails avoidance behaviour standardized tests (ISO, 2008, 2010). Avoidance behaviour, first applied to evaluate earthworm behaviour in escaping from contaminated soils (Yeardley et al., 1996), have been used to evaluate the effects of pollutants to several soil organisms (Amorim et al., 2008; Natal-da-Luz et al., 2008; Loureiro et al., 2009; Owojori and Reinecke, 2009). These tests are nowadays considered a good tool to screen soil contamination since they are cheap, quick and easy to perform and furthermore are sensitive to a broad spectrum of contaminants (Röembke, 2008). In conclusion, avoidance behaviour represents a robust endpoint to evaluate and predict the effects of environmental contamination in soil organisms (Loureiro et al., 2005; Aldaya et al., 2006).

Among the several parameters studied in ecotoxicological tests, the evaluation of the reproduction output of soil organisms is considered to be an effective and ecological relevant parameter to assess pesticide effects in terrestrial ecosystems. Thus, to assess the effects of pesticides in soil organisms standardized ecotoxicological laboratory tests mainly focused on parameters like survival and/or reproduction are available (e.g. ISO, 1999).

The herbicide glyphosate inhibits the biosynthesis of aromatic amino acids, and deregulates the shikimate pathway which leads to general metabolic disruption (Reddy et al., 2008). The insecticide dimethoate is one of the most commonly applied insecticides in agricultural fields, which acts trough the inhibition of cholinesterase enzyme activity (Martikainen, 1996). Spirodiclofen is a selective, non-systemic acaricide from the novel class of tetronic acid derivatives, which interferes with lipid biosynthesis by inhibiting acetyl-Coa carboxylase (Wachendorff et al., 2002; Nauen, 2005). The three plant protection products (PPPs) were chosen based on their different modes of action, but also due to the market share that dimethoate and glyphosate have in Europe as the most sold pesticides and spirodiclofen, which has been recently introduced in Portugal (Vieira, 2009).

Considering the possible and real scenarios of fields contaminated with PPPs cocktails and the available tools to evaluate their potential effects on key-species populations, the purposes of this study were: firstly, to determine the effects of three commonly used PPPs on the avoidance response pattern of *P. pruinosus* and in the reproductive output of *F. candida*; secondly to predict the response patterns for mixture exposures using the CA and IA conceptual models for the two test-species.

2. Materials and methods

2.1. Test organisms

The isopods used in these experiments were obtained from a laboratorial culture, where they are maintained in a climatic chamber at 25 °C, 60% moisture content, and with a 16 h:8 h light:dark photoperiod. Only adult animals (15–25 mg wet weight) were used. No sex differentiation was done, although pregnant females were not chosen to the experimental procedure.

The collembolan were obtained from a laboratory culture, maintained at a constant regime of 16 h light, 8 h dark and a constant temperature of 17 ± 2 °C. The springtails were cultured in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 10:1. On a weekly basis granulated dry yeast was added as food in small amounts to avoid spoilage by fungi.

2.2. Test chemicals and test soil

Three pesticides were used in the experimental procedure as commercial formulations: the post-emergence herbicide glyphosate (ROUNDUP® with 360 g AI L^{-1}, and which contains glyphosate-isopropylammonium (45%), surfactant (16%) and water (42.5%)), the organophosphorous insecticide dimethoate (AGROR® with 400 g AI L^{-1} and which contains dimethoate (40%), ciclohexanone (28.4%), nonylphenol ethoxylate (2.2%), petroleum naphta (26.1%) and calcium alkyl benzene sulphonate inpropil 2-ol (0.4%)) and the acaricide spirodiclofen (ENVIDOR® with 240 g AI L^{-1} which contains spirodiclofen (23.3%), ethoxylated polyarylphenol (1–22.5%) and glycerine (>1%)). The nominal concentrations used for glyphosate ranged from 0.5 to 54.5 mg kg^{-1} dry soil in the avoidance experiment and between 0.1 and 2 mg kg^{-1} dry soil in the reproduction test; for dimethoate the nominal concentration used ranged from 0.2 to 80 mg kg^{-1} dry soil in the avoidance experiment and between 0.1 and 2 mg kg^{-1} dry soil in the reproduction experiment; for spirodiclofen the nominal concentration used ranged from 0.04 to 2.7 mg kg^{-1} dry soil in the avoidance and reproduction tests.

All tests were performed with LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer. The properties of this soil include a pH = 5.8, organic matter = 3.9%, texture = 6% clay; 17% silt and 77% sand.

2.3. Single exposure procedure with P. pruinosus

The avoidance tests conducted with *P. pruinosus* were performed based on a methodology proposed by Loureiro et al. (2005), consisting in exposing 10 isopods in a rectangular plastic box (14.3 cm × 9.3 cm × 4.7 cm height) divided in two sections, one with the control soil and the other with the test soil (contaminated). Five concentrations of each pesticide plus the double control (both sides with control soil) were tested using three replicates each, in a total of 18 test boxes per pesticide (Fig. 1). The volume of pesticides needed was added to distilled water and spiked in the soil (adjusted to 60% of the WHC). The pH was measured in the beginning and end of the test, and no significant changes in pH values were observed in any of the concentrations tested (pH values varied in a 5% interval). After 24 and 48 h the number of animals in each side of the test-box was counted and mortality was registered. The percentage of avoidance was calculated using the formula $A = \frac{C-T}{N} \times 100$ (ISO, 2008), where A is the percentage of avoidance, C is the number of animal in control soil, T is the number of isopods in test (contaminated) soil and N is the total number of organisms. All calculations done were based in the nominal concentrations. The values of percentage of avoidance were used to calculate the AC_{50} in the several single exposures, and subsequently to design the binary mixture combinations.

2.4. Single exposure procedure with F. candida

The experimental procedure for the reproduction test with the springtail *F. candida* was performed accordingly to the ISO 11267 protocol (ISO, 1999). In the beginning of each experiment, 30 g of sieved soil (dry weight; 5 mm mesh) was moistured to 60% of its water holding capacity. Five concentrations of each pesticide plus the control were tested using five replicates each, in a total of 30 test boxes per pesticide. Afterwards, 10 synchronised springtails (10–12 d old) were introduced in each of the five replicates. In the beginning of the test and after a 14 d period, approximately

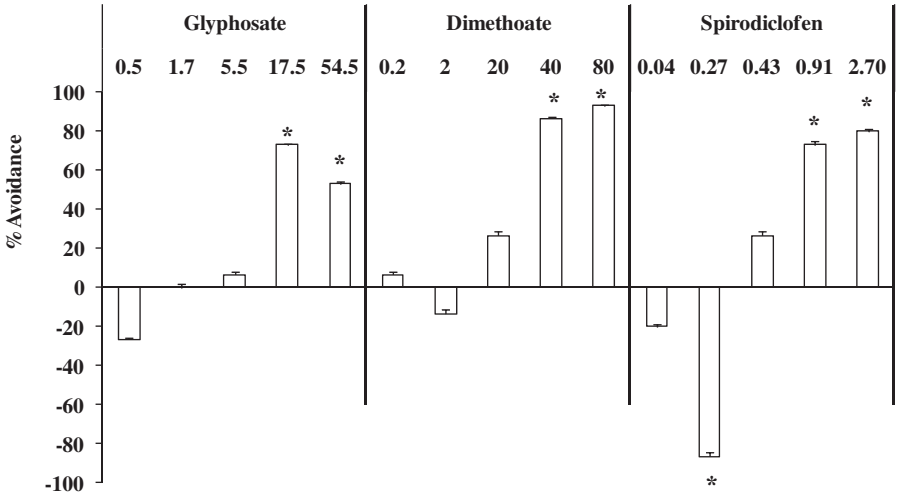

Fig. 1. Avoidance response of *Porcellionides pruinosus* in LUFA 2.2 soil after application of glyphosate, dimethoate and spirodiclofen (mean net response with standard error bars). All units (nominal values) are in mg Al kg^{-1} dry soil.

2 mg of dry yeast was added to all test containers. Twice a week the tests containers were opened to allow aeration, and water was added when necessary. After 28 d, the content of each test vessel was carefully transferred into larger vessels and filled up with water and some drops of blue ink. After moderate stirring, adults and juveniles floating in the water surface were photographed and counted using the image analysis software provided by Sigma Scan. The pH was measured in the beginning and end of the test, and no significant changes in pH values were observed in any of the concentrations tested (pH values varied in a 5% interval).

2.5. Mixture exposure

In the mixture experiments 23 binary combinations, based on the AC_{50} and EC_{50} values found in the previous single exposure experiments, were made simultaneously with five single concentrations of each pesticide and one control, without replication, in a total of 34 test boxes for each binary experiment (Figs. 3 and 4). The range of toxic units (1 TU = EC_{50} or AC_{50} of the pesticide) in the binary experiments went from 0.375 to a maximum of 3 (see Figs. 3 and 4 for further details). The TU of each pesticide was never higher than 2 in any of the binary combinations made. In the mixture experiments, the number of replicates per combination was reduced to one, so that more combinations could be tested and thus a wider set of points along the response surface could be obtained (Ferreira et al., 2008). As a consequence the power of the analysis increased since the analysis performed lays on regression models and differences calculated between the data obtained and the modeled values (Jonker et al., 2005).

2.6. Statistical analysis

For the avoidance behaviour tests the concentration causing an avoidance percentage of 50% in exposed animals (AC_{50}) for each pesticide was calculated using the Probit regression scheme (MINITAB 13), assuming that when 50% of the animals are in each section of the box, no avoidance effect is observed ($A = 0$).

For the reproduction test, differences between the number of juveniles produced by collembolans in the control and the soil treatments were analysed using a one-way ANOVA, followed by

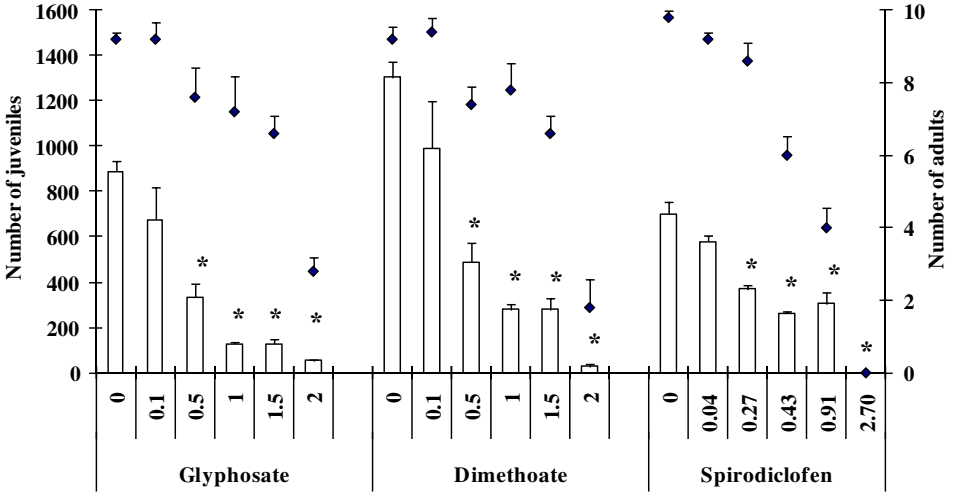

Fig. 2. Reproductive output and effects on survival on *Folsomia candida* (mean number of juveniles + standard error bars) exposed to glyphosate, dimethoate and spirodiclofen spiked in the LUFA 2.2 soil. White bars indicate data for juvenile production (mean values and st. error) and black dots indicate adult survival (mean values and st. error) after the 28 d exposure. All units (nominal values) in mg Al kg^{-1} dry soil. (* indicates statistical differences for offspring production (Dunnett's method, $p < 0.05$)).

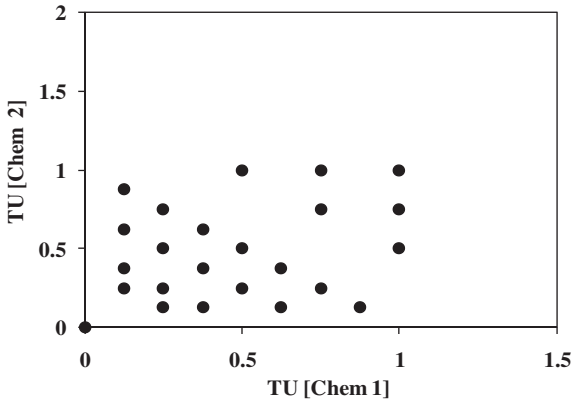

Fig. 3. Experimental design of the binary combinations performed in the reproduction tests with *Folsomia candida* based on the toxic units (1 TU = EC_{50}) of glyphosate, dimethoate and spirodiclofen obtained in the single exposure tests. Data for [Chem 1] vs. [Chem 2] refers to glyphosate vs. dimethoate; glyphosate vs. spirodiclofen; dimethoate vs. spirodiclofen.

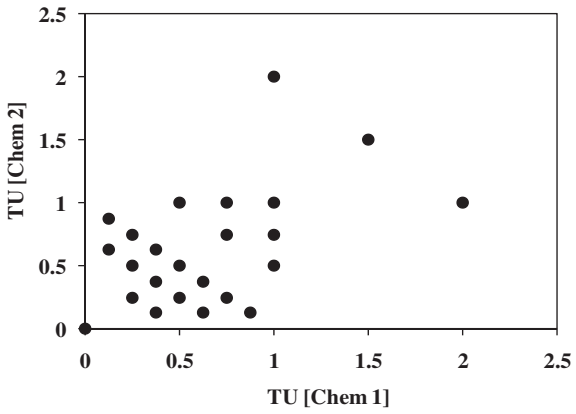

Fig. 4. Experimental design of the binary combinations performed in the avoidance tests with *Porcellionides pruinosus*. based on the toxic units (1 TU = EC_{50}) of glyphosate, dimethoate and spirodiclofen obtained in the single exposure tests. Data for [Chem 1] vs. [Chem 2] refers to glyphosate vs. dimethoate; glyphosate vs. spirodiclofen; dimethoate vs. spirodiclofen.

post hoc Dunnett's test ($\alpha < 0.05$). EC_{50} values were determined using appropriate (i.e. the best fit) non-linear models. The concentration responsible for a decrease in 50% of adult collembolans that survived the exposure period (LC_{50}) was calculated following the same procedure. For all the analysis mentioned above, STATISTICA 7.0 software provided by StatSoft Inc., was used.

The tool used to analyze and compare the data in the present study was the MixTox model (Jonker et al., 2005), which allow us to fit data to both reference models (CA and IA), thus comparing the observed toxicity and the expected toxicity of the pesticides and also to calculate possible deviations from the two reference models. These deviations are given by quantitative parameters (*a* and *b*) that can express a higher effect than expected (synergism) or a smaller effect than expected (antagonism) by the reference models (see Table 1 for further information). Two more complex deviations can also be obtained, depending on the level of the pesticides in the mixtures (dose level dependence deviation) or on the composition of the mixture (dose ratio dependence deviation).

3. Results

3.1. Single exposure procedure – P. pruinosus

In the double control the animal were randomly distributed through both sides of the test-box, showing no preference for one side of the box in detriment of the other side. In two replicates of glyphosate and spirodiclofen one isopod was found dead after the 48 h period. In the mixtures tested no mortality was observed.

The exposure to glyphosate resulted in a clear avoidance response in the higher concentrations of the herbicide (73% avoidance at 17.4 mg kg^{-1}) although a small decrease in the degree of avoidance response was reflected in the highest concentration (Fig. 1 and Table 2). Dimethoate caused a clear avoidance response by the isopods (Fig. 1), and in the two highest concentrations

Table 2
AC_{50} values (mg kg^{-1} dry soil) and 95% confidence intervals (CI) for the effect of single exposure pesticide on the avoidance behaviour of *Porcellionides pruinosus* exposed for 48 h on LUFA 2.2 soil. Values are derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	AC_{50} (95% CI)
Glyphosate	39.7 (34.7–46.2) (single exposure experiment) 16.8 (12.9–21.1) (mixture experiment with dimethoate) 42.9 (39.7–47.1) (mixture experiment with spirodiclofen)
Dimethoate	31.5 (28.4–34.9) (single exposure experiment) 31.3 (23.4–39.8) (mixture experiment with glyphosate) 43.1 (37.0–50.2) (mixture experiment with spirodiclofen)
Spirodiclofen	0.9 (0.7–1.1) (single exposure experiment) 1.5 (1.0–2.1) (mixture experiment with dimethoate) 1.9 (1.7–2.1) (mixture experiment with glyphosate)

Table 1
Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from the reference models concentration addition (CA) and independent action (IA); adapted from Jonker et al. (2005).

Deviation pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
Synergism/antagonism (S/A)	$a > 0$: antagonism $a < 0$: synergism		
Dose ratio dependent (DR)	$a > 0$: antagonism except for those mixture ratios where negative *b* value indicate synergism $a < 0$: synergism except for those mixture ratios where positive *b* value indicate antagonism	$b_i > 0$: antagonism where the toxicity of the mixture is caused mainly by toxicant *i* $b_i < 0$: synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose level dependent (DL)	$a > 0$: antagonism low dose level and Synergism high dose level $b_{DL} = 1$: change at EC_{50} level $a < 0$: synergism low dose level and antagonism high dose level	$b_{DL} > 1$: change at lower $b_{DL} = 1$: change at EC_{50} level $0 < b_{DL} < 1$: change at higher EC_{50} level $b_{DL} < 1$: no change but the magnitude of S/A is DL dependent	$b_{DL} > 2$: change at lower EC_{50} level EC_{50} level $b_{DL} = 2$: change at EC_{50} level $1 < b_{DL} < 2$: change at higher EC_{50} level $b_{DL} < 1$: no change but the magnitude of S/A is effect level dependent

tested (40 and 80 mg kg^{-1}) the percentage of animals in the control soil were, respectively, 87% and 93% (Fig. 1 and Table 2). A strong avoidance effect was observed after the exposure of *P. pruinosus* to spirodiclofen (Fig. 1 and Table 2). Although in the concentration 0.27 mg kg^{-1} more isopods were found in the contaminated side, when doses of spirodiclofen increased a clear avoidance response pattern was observed.

3.2. Single exposure procedure – F. candida

The number of adult collembolans that survived the experimental procedure diminished with increasing dosages of glyphosate. This exposure resulted also in a clear reduction in the number of juveniles produced (Fig. 2). In the two highest concentrations of this herbicide a reduction in 86% and 95% in offspring production was observed. The LC_{50} calculated for glyphosate was six times higher than the value for the EC_{50} (Table 2). In the highest concentration tested a 98% reduction in the number of juveniles produced was observed due to the high impact in the survival rate of collembolan exposed to dimethoate The LC_{50} calculated for dimethoate was four fold the value of the EC_{50} (Table 2). The exposure of *F. candida* to spirodiclofen resulted in a decrease in both survival and reproduction rates with increasing dosages of this insecticide (Fig. 2). In the highest concentration of this insecticide there was a reduction in 100% of the adults that survived the test period. The LC_{50} calculated for spirodiclofen was similar to the one obtained in the EC_{50} calculation (Table 2).

Table 3
EC_{50} values (mg kg^{-1} dry soil) and 95% confidence intervals (CI) for the effects of single exposure pesticides on the reproductive output of *Folsomia candida* exposed for 28 d on LUFA 2.2 soil. Values are derived from single chemical exposure in tests run simultaneously with the mixture exposure.

Pesticide	EC_{50} (95% CI)
Glyphosate	0.33 (0.18–0.48) (single exposure experiment) 0.15 (0.02–0.28) (mixture experiment with dimethoate) 0.1 (0.07–0.14) (mixture experiment with spirodiclofen)
Dimethoate	0.37 (0.19–0.54) (single exposure experiment) 0.05 (0.03–0.07) (mixture experiment with glyphosate) 0.15 (0.08–0.21) (mixture experiment with spirodiclofen)
Spirodiclofen	0.65 (0.37–0.96) (single exposure experiment) 0.27 (0.15–0.39) (mixture experiment with glyphosate) 0.44 (0.05–0.92) (mixture experiment with dimethoate)

3.3. Mixture exposure – P. pruinosus

Data from the combination of glyphosate and spirodiclofen was fitted to the CA model, which resulted as the best descriptive model (Table 4) But when the same data were fitted to the IA model, a dose level dependent deviation from the reference conceptual model was found with the negative parameter *a* indicating that synergism occurs at low dose levels of both pesticides and antagonism occurs at higher doses of both pesticides. The parameter *b* was higher than 1, thus indicating that the change between synergism and antagonism takes place at lower doses than the AC_{50} of the pesticides (Table 4).

For the dimethoate and glyphosate exposure experiment, after fitting the data to the CA model an antagonistic deviation from the reference model was observed by the positive parameter *a*, meaning that the predicted escape response of the animals was lower than the expected considering their individual response. After fitting the data to the IA model, the most parsimonious result was the reference model and no deviations were observed (Table 4).

Data from the combined experiment of dimethoate and spirodiclofen fitted to both reference models, translated into an antagonistic deviation. After fitting the data to the CA model, antagonism was observed (Table 4). After using the IA model, an antagonistic pattern was also observed as the parameter *a* was also higher than zero (Table 4).

3.4. Mixture exposure – F. candida

After fitting the data from the combination of glyphosate and spirodiclofen to the CA model a deviation for antagonism was found (Table 4) The same antagonistic deviation was observed when this data was fitted to the IA conceptual model since once again the parameter *a* was higher than zero (Table 4).

For the dimethoate and glyphosate exposure experiment, after fitting the data to the CA model an antagonistic deviation from the reference model was observed, with a positive parameter *a* meaning that the predicted toxicity of the mixture was lower than the expected considering the individual exposure effects. After fitting the data to the IA model, the same result was obtained, i.e. a deviation for antagonism (Table 5).

When data of the binary experiment of dimethoate and spirodiclofen was fitted to the CA model, the most parsimonious result was the reference model. After fitting our data to the IA model, the most parsimonious model was also the reference model itself

Table 4
Parameter estimates and tests of fit of the reference models using the MixTox model applied to the behavioural avoidance response of *Porcellionides pruinosus* exposed for 48 h to three pesticide mixtures in LUFA 2.2 soil.

Mixture		Concentration addition					Independent action				
		r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*
Glyphosate and spirodiclofen	Reference	0.19	***	100.6	–	–	–	–	–	–	–
	S/A	–	–	–	–	–	–	–	–	–	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	0.24	*	93.1	−19.8	115.9
Dimethoate and glyphosate	Reference	–	–	–	–	–	0.23	**	61.3	–	–
	S/A	0.27	*	58.3	4.5	–	–	–	–	–	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	–	–	–	–	–
Dimethoate and spirodiclofen	Reference	–	–	–	–	–	–	–	–	–	–
	S/A	0.46	*	22.1	7.1	–	0.48	*	21.2	5.2	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	–	–	–	–	–

r^2 is the coefficient of determination; $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance levels: *<0.05; **<0.01; ***<0.001); SS is residuals sum of squares; *a* and *b* are the parameters of the deviations; S/A is synergism/antagonism, DR is dose ratio deviations and DL is dose level deviation from the reference models.

and no deviations were obtained, since subsequent addition of extra parameters did not improve the fitness of the model (Table 5).

4. Discussion

4.1. Single exposure toxicity – P. pruinosus

The AC_{50} calculated from the single exposure procedure and the single exposures in the combined experiments for the three pesticides were in the same range of values for the three pesticides tested (Table 2). In fact, in only two combinations (glyphosate with dimethoate and spirodiclofen with dimethoate) the values were twofold the ones calculated in the single exposure procedure.

Single exposure to the herbicide glyphosate caused a clear avoidance response in the terrestrial isopod *P. pruinosus*. In a laboratory study where the direct mortality of the isopod *Philoscia muscurom* exposed to glyphosate application rates of 6 L ha^{-1} (≈2.1 kg AI ha^{-1}) evident effects on survival rate were observed (Eijsackers, 1985) and in a subsequent study, a decrease in the consumption rate of leaf litter treated with the same amount of herbicide was also observed (Eijsackers, 1991). In a laboratory study with the earthworm *Aporrectodea caliginosa* treated at levels equivalent to application rates of 0.7–2.8 kg glyphosate ha^{-1}, a decrease in growth rates and early mortality of the earthworms were observed (Springett and Gray, 1992). In the present study the concentration that caused an avoidance response in *P. pruinosus* was almost 17 times higher than the predicted environmental concentration (PEC) in the top 10 cm soil (3060 g AI ha^{-1} ≈ 2.5 mg kg^{-1} dry soil) if the commercial formulation is applied at labelled doses.

Previous studies with the same isopod species and dimethoate obtained very similar AC_{50} values as these reported here, in individual (39.43 mg kg^{-1}) and collective (28.67 mg kg^{-1}) avoidance tests (Loureiro et al., 2005). Another study dealing with the influence of dimethoate to the isopod *Porcellio scaber* (Engenheiro et al., 2005) observed a reduction in 50% of the survival rate of this organism after 10 d of exposure to 20 mg kg^{-1}. According to the same study dimethoate clearly influenced the locomotor behaviour of *P. scaber*, related with a significant decrease in acetylcholinesterase (AChE) activity, since this OP insecticide is a well known inhibitor of this nervous system enzyme (Bayley, 1995). The results obtained in the present study seem to indicate that this insecticide exerts a clear dose-related behavioural pattern in *P. pruinosus*. However it should be noted that the PEC value (400 g AI ha^{-1} ≈ 0.3 mg kg^{-1} dry soil) is much lower than the concentrations causing an avoidance response in this species. In the two highest dimethoate concentrations tested, more than 80% of the animals were found in the control side of the test boxes. This situation represents a loss in the "habitat function" of the soil (Hund-Rinke et al., 2003) and should be taken into account in evaluating the detrimental effects in isopods population after dimethoate application in agricultural fields. The effects of dimethoate on the species *P. scaber* have also been assessed in terms of mortality (Løkke and Van Gestel, 1998) and growth pattern (Fischer et al., 1997), and the values obtained for these two parameters, 75 mg kg^{-1} and 17.5 mg kg^{-1}, respectively, can be considered in the same range of the AC_{50} values reported here.

Our results indicated that spirodiclofen had an impact in the avoidance behaviour of *P. pruinosus*, being the calculated AC_{50} much lower (0.91 mg kg^{-1}) than the values obtained for the other two pesticides tested. Nevertheless it should be pointed out that the AC_{50} is more than 10 times higher than the predicted environmental concentration in the top 10 cm soil (PEC) if spirodiclofen is applied at recommended field rates (96 g AI ha^{-1} ≈ 0.06 mg kg^{-1} dry soil). At 0.27 mg spirodiclofen kg^{-1} dry soil, which is below spirodiclofen response threshold, more animals were found in the contaminated side of the box. Possibly this could be related with the attraction that some substance of the formulation exerted on isopods (Olla et al., 1980; Zimmer et al., 1996). This finding (non-avoidance of contaminated soil) has been described in other experiments with non-narcotic chemicals (Yeardley et al., 1996; Odendaal and Reinecke, 1999) and with narcotic chemicals (Heupel, 2002; Landrum et al., 2003) in different test-species.

After an application rate of the commercial formulation Envidor® in an open field, spirodiclofen was considered very toxic to *Tetranychus urticae* and moderately toxic to *Tarsodemus pallidus* (Raudomis, 2006). All these results seem to confirm the toxicity of this insecticide, thus attention should be paid in analyzing possible damages in non-target organisms like the macro decomposers terrestrial isopods, following the application of this product to agricultural crops.

4.2. Single exposure toxicity of F. candida

The EC_{50} value calculated for the insecticide dimethoate (in the single experiments performed simultaneously to the binary combinations) was six times lower than the value obtained in the single exposure procedure, although the value for glyphosate can be considered in the same range of values obtained in the single exposure procedure (Table 3). The same can be said about the insecticide

Table 5
Parameter estimates and tests of fit of the reference models using the MixTox model applied to the reproductive output of *Folsomia candida* exposed for 28 d to three pesticide mixtures in LUFA 2.2 soil.

Mixture		Concentration addition					Independent action				
		r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*
Glyphosate and spirodiclofen	Reference	–	–	–	–	–	–	–	–	–	–
	S/A	0.68	***	395501.1	3.9	–	0.67	**	399954.6	3.9	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	–	–	–	–	–
Dimethoate and glyphosate	Reference	–	–	–	–	–	–	–	–	–	–
	S/A	0.61	*	477742.1	2.3	–	0.64	*	438094.4	2.9	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	–	–	–	–	–
Dimethoate and spirodiclofen	Reference	0.77	***	372975.3	–	–	0.76	***	400299.9	–	–
	S/A	–	–	–	–	–	–	–	–	–	–
	DR	–	–	–	–	–	–	–	–	–	–
	DL	–	–	–	–	–	–	–	–	–	–

r^2 is the coefficient of determination; SS is the residuals sum of squares; $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance levels: *<0.05; **<0.01; ***<0.001); *a* and *b* are the parameters of the deviations; CA is concentration additions; IA is independent actions; S/A is synergism/antagonism, DR is dose ratio deviations and DL is dose level deviation from the reference models.

spirodiclofen, since the EC_{50} values obtained were in the same range of concentration.

The effects of the application of the herbicide glyphosate to collembolan populations have been assessed in field plot experiments. One study showed that the application of this herbicide favoured the appearance of epigeic and hemiedaphic species due to the preservation of weed cover given by glyphosate (Renaud et al., 2004). A previous study has also demonstrated the indirect effect of this herbicide on the enhancement of biological activity in soils, since the supply of organic matter (due to the killing of the cover crop) had a positive influence on the feeding activity of soil microbial communities (Reinecke et al., 2002). However, other studies dealing with different herbicide and collembolan species showed contrasting results. A field and laboratory test observed that atrazine reduced the abundance of *Entomobrya musatica* (Al-Assiuty and Khalil, 1998) at doses near the recommended application rate of this herbicide. Another study also detected effects on the reproductive capacity of collembolan at herbicides concentrations below the mortality threshold (Chernova et al., 1995). The impairment in the reproductive output of *F. candida* detected in the present work clearly indicates that the application of this herbicide does have an impact in both the survival and the reproductive capacity of this collembolan species.

The effects of the insecticide dimethoate on the reproduction of the collembolan *F. candida* have been assessed previously. Krogh (1995) have found that the application of dimethoate near the recommended dose had an impact in the reproductive output (EC_{50} of 0.5 (0.4–0.6) mg kg^{-1}) and survival rate of adults (LC_{50} of 0.6 (0.6–0.6) mg kg^{-1}). Another work dealing with the same insecticide applied to three different soil types (Martikainen, 1996) derived EC_{50} values between 3.8 and 6.3 mg kg^{-1}, which were attributed to the high organic content of the soil used, responsible for the reduction of dimethoate toxicity. More recent studies have attested dimethoate toxicity and its impact on the reproductive capacity of *F. candida* (Sørensen and Holmstrup, 2005). The capacity to predict the effect of pollutants in dynamic natural population can be regarded as the main objective of ecotoxicological studies (Moe et al., 2001), so it seems pertinent to address the question if dimethoate could in long term exposure periods cause a disruption in the population dynamics of this collembolan species.

Since the DT_{90} of spirodiclofen and its soil metabolites is less than 100 d no testing with soil non-target macro-organisms was triggered for regulatory purposes, however this pesticide was considered of low risk to earthworms and to the collembolan species *F. candida* when applied at recommended doses in agricultural fields (Candolfi et al., 2001). The EC_{50} calculated for spirodiclofen was 0.65 (0.37–0.96) mg kg^{-1}, which represents 10-fold of the PEC if Envidor® is applied at the labelled recommended dose. It should be noted, however, that the field soil dissipation of this product is considered to be very fast (DT_{50} between 0.5 and 5.5 d), thus in agricultural systems the period in which this product is available should be taken into account when inferring the effects this pesticide may have to edaphic organisms.

4.3. Mixture exposure toxicity

4.3.1. Mixture exposure toxicity – P. pruinosus

Of the three binary combinations tested only when the two insecticides, dimethoate and spirodiclofen, were applied together the same deviation (antagonism) was obtained from the two reference models. In the other two binary combinations the predictions were not the same, although they were not discordant, in view of the fact that in both cases CA or IA did not predict a deviation from the conceptual models.

The exposure to the combination of glyphosate and spirodiclofen followed the conceptual model of CA, hence their combined effects produce a predictable response based on the measured effects of single chemicals (Faust et al., 1993). This simple additive effect was also registered in an experiment performed with mixtures of an herbicide and an insecticide (atrazine and lindane) in a freshwater microcosm (Van den Brink et al., 2009), where it was concluded that the toxic effects of the binary mixture could be explained by the effects of the individual chemicals alone.

After fitting the IA model to the same data, a dose level deviation was found, indicating synergism at low doses of the two pesticides and antagonism with increasing doses (higher than the respective AC_{50}) of the two pesticides. This means that the simple additive effect predicted by CA was not corroborated by the IA model; instead a deviation from this reference model was detected, hence the probability of response (avoiding the contaminated soil) to the two chemicals was not independent. Synergism found at low doses of the two pesticides is an indication of higher escape response than expected from the individual action of both chemicals, and thus may represent an hazard for the communities exposed to the two pesticides.

Dimethoate and glyphosate behavioural data when fitted in the CA model translated into antagonism, predicting a smaller effect of the two pesticides than expected. When this data was fitted in the IA model, it followed the conceptual model attesting the *a priori* knowledge that these pesticides have dissimilar modes of action. A previous study performed with the isopod *P. pruinosus* with binary mixtures of dimethoate and the triazine herbicide atrazine predicted antagonism at low doses of both pesticides and a switch to synergism at high doses of both pesticides after fitting data to the IA model (Loureiro et al., 2009). This deviation is in agreement with the antagonistic effect found in the present work, although predicted by the other reference model used in mixture toxicity assessment.

Regarding the same mixture data, no deviation from the conceptual model of IA was found in the same study when this reference model was applied. Previous studies dealing with binary mixtures of atrazine and OP insecticides have detected a synergistic effect of the herbicide to the toxicity of chlorpyrifos and diazinon to the aquatic organisms *Chironomus tentans* and *Hyalella azteca* (Anderson and Lydy, 2002; Jin-Clark et al., 2002). Similar studies performed with *C. tentans* midges found that the dimethoate toxicity is enhanced due to the oxidative activation process brought by the action of the herbicide atrazine on the cytochrome P450 monooxygenase (Cyt-P450) enzymatic complex (Anderson and Zhu, 2004). This molecular activation of dimethoate due to the action of the herbicide atrazine causes a cascade of reactions that culminate in the increase inhibition of AChE activity, resulting in a higher toxicity of the two pesticides to the chironomid midges. In the present work, apparently, this molecular activation of the insecticide dimethoate did not occur, and this can be explained by the different molecular mechanism of action of the herbicide glyphosate in comparison with atrazine (although known only for plants). The same synergistic effect was found in a more recent work with the same test-species, where the action of two chloroacetanilide herbicides (alachlor and metolachlor) on chlorpyrifos was observed (Jin-Clark et al., 2008). These herbicides reduced the activity of the detoxifying enzyme glutathione-S-transferase (GST) which led to the interruption of metabolic detoxification of chlorpyrifos, thus increasing the quantity of this insecticide inside the organism and consequently increasing the peril of intoxication.

The effect of dimethoate and spirodiclofen on *P. pruinosus* avoidance behaviour showed an identical deviation from the two reference models, since in both models antagonism was predicted. The combined effect of dimethoate and the organochlorine insecticide lindane was also assessed for this isopod species, and deviations from the two reference models were also observed (Loureiro et al., 2009). In the cited work, it was observed that after

fitting the avoidance data of dimethoate and lindane to CA model a dose ratio dependency did occur, with higher toxicity than expected found when lindane was the dominant component in the mixture; but when the same data was fitted into the IA conceptual model a dose level deviation was observed, with antagonism at low doses and synergism with increasing doses (higher than the AC_{50}) of both pesticides. The prediction obtained when testing the IA model was in agreement with the results found in the present work, given that antagonism was detected at smaller doses of both pesticides and the synergism observed was registered only at higher levels of both pesticides, thus its biologically relevancy can be questioned since at higher doses the animal could not survive the exposure concentrations.

4.3.2. Mixture exposure toxicity – F. candida

There was a decrease in the number of juveniles with increasing toxicity of the binary combinations tested, but the degree in which the number of juveniles diminished was not as high as expected by the results obtained in the single exposure procedures run simultaneously. Individual concentrations of glyphosate and spirodiclofen originated a strong and abrupt decrease in the number of juveniles produced (reduction in 90% of the juveniles produced in doses higher but near the EC_{50} in comparison to the control), but when the same concentrations were tested in the binary mixtures (sum of TU between 1 and 2) the reduction in the number of juveniles compared to the control was between 70% and 60% in the mixture of glyphosate and spirodiclofen. The same happened in the binary mixtures of dimethoate and glyphosate, since when both toxicants were individually present at concentrations higher but near the respective EC_{50} the decrease in the number of juveniles was between 85% and 90% of the offspring observed in control, whereas in the binary combinations with correspondent concentrations the decrease was between 85% and 50% in comparison to the control. As a consequence of this decrease that was not as steeper as the expected, and regarding the observed individual concentrations, antagonism was predicted for these two binary mixtures.

Glyphosate enhanced both glutathione-S-transferase (GST) and superoxide dismutase (SOD) enzymatic activity in the aquatic oligochaete *Lumbricus variegatus* submitted to a commercial formulation of this herbicide (Jara et al., 2009). The two antioxidant enzymes that are related with detoxifying xenobiotics and cellular stress were both activated at non-toxic levels of this herbicide. In a study with a selected strain of the two spotted spider mite *T. urticae*, it was observed that increased GST and cyt-P450 activity led to a spirodiclofen resistance factor of 13 (Rauch and Nauen, 2003). This could conduct us to hypothesize the possible biochemical interactions between the two pesticides, since the enhanced activity caused by glyphosate in the cited enzymatic complexes could lead to an increasing resistance to spirodiclofen toxic effects in the target organism. A work testing the efficacy of IA in describing several dissimilar binary combinations demonstrated that IA was the most parsimonious model to describe 6 out of the 10 combinations tested, but antagonism was observed in the binary combination of chlorpyrifos and the fungicide inhibitor of cyt-P450 prochloraz (Martin et al., 2009). This was stated to be a result of the inhibition of the cyt-P450 complex by the fungicide, which could lead to an inactivation of chlorpyrifos into its more toxic form. Again, there was a correlation between biochemical changes in enzymes involved in cellular stress caused by one chemical and on subsequent magnitude of insecticide toxicity to organisms.

A previous study dealing with the combined effect of the herbicide atrazine with five different OP insecticides and one organochlorine insecticide to aquatic midge larvae (*C. tentans*) was evaluated using the IA model (Pape-Lindstrom and Lydy, 1997). Their results predicted synergism (effects greater than additive) in four of the OP insecticides (chlorpyrifos, malathion, methyl-parathion, and trichlorfon) and antagonism when the herbicide was mixed with mevinophos. In that work a consistent and significant enhancement in toxicity of the majority OP insecticides due to the action of atrazine was observed, and several hypotheses were raised from an increasing cellular permeability of the midge cuticle caused by atrazine to the activation of cyt-P450 by the herbicide. An acute experiment (filter-paper test) made with the earthworm *Eisenia fetida* (Lydy and Linck, 2003) found that when chlorpyrifos was mixed with atrazine and cyanazine these herbicides increased the toxicity of chlorpyrifos by a factor of 7.9 and 2.2, respectively. Still, when chlorpyrifos was mixed with symazine this herbicide did not affect the insecticide toxicity, probably as a result of a non-activation of the insecticide. In an experiment carried out in the field, mite and ant populations were reduced after application of herbicides (nicosulfuron and atrazine) mixed with chlorpyrifos (Pereira et al., 2005). However, an opposite conclusion was derived from exposure of the larvae of *Cacopsylla pyri* to atrazine where this herbicide led to a decrease in mortality following an exposure to the insecticide *Bti* (Boyer et al., 2006).

From the studies described above one must admit that further knowledge of the pesticides mode of toxic action should be established to make more assertive assumptions about the mechanisms involved in the combined toxicity of herbicides and insecticides to soil organisms. In the lack of systematic understanding of the mechanisms underlying the toxic effects on the specific test organism, only general considerations could be made about mixture toxicity interaction inside the target organisms.

The mixture of dimethoate and spirodiclofen followed the two conceptual models when data was fitted to CA and IA. This means that the effects obtained when these two insecticides were applied jointly could be expressed as simple dilution of each other (after CA conceptual model), implying that one insecticide could be replaced by a dilution of the other without alterations in the resultant toxicity (Berenbaum, 1989). The same result was obtained in previous studies dealing with the effects of insecticides of similar action (AChE inhibitors dimethoate and pirimicarb) on *Daphnia magna*, where the binary mixture followed CA, but IA could also explain the data reasonably well (Syberg et al., 2008).

4.4. Comparison between avoidance and reproduction tests

Although the two tests evaluated different test-species, different parameters (avoidance behaviour and reproduction output) and different exposure periods a comparison between the outcome of the two test should be established. Looking at the AC_{50} values of glyphosate and dimethoate the median values were more than 100-times higher than the EC_{50} calculated for the collembolan reproduction. Only in the case of spirodiclofen the values can be considered in the same range interval. The discrepancy between the AC_{50} and EC_{50} values could be explained by the different sensitivity to PPPs application, since the isopods move mainly in the soil surface and have clean (uncontaminated) soil to where they can escape and collembola are buried in contaminated soil during the test period. In addition, avoidance test often gave higher ECx values than reproduction chronic tests (Amorim et al., 2005), which could be related with the short period used in avoidance test (48 h) in comparison to the 28 d in reproduction tests. Thus avoidance behaviour tests can be used as initial screening test in soil contamination assessment and not as a substitute of reproduction tests (Loureiro et al., 2005).

5. Conclusions

From an overall observation of the results obtained from fitting the data of avoidance behaviour and reproductive output to both

models several assumptions can be made regarding the ability of the two conceptual models in predicting mixture toxicity. Data from the mixtures followed the reference models in 4 out of the 12 combinations tested (see Tables 3 and 4). In 7 out of the 12 mixtures performed the two reference models predicted antagonism. In only one combination (glyphosate and spirodiclofen applied to *P. pruinosus*) synergism was observed. There was a general agreement between the two reference models in predicting mixture toxicity, with the exception of two mixtures tested. As a result of this, both models could and should be used to address mixture toxicity problems, since the information gathered from fitting data to both models was complementary and never contradictory, in order to understand what happens when plant protection products are applied together.

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